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The effect of hyperglycemia on the macrophages in the cell culture

Running head: Hyperglycemia effect on macrophages

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Abstracts

Background: The availability of glucose in tissue has a role in macrophages polarization into an inflammatory phenotype. The over nutrition condition such as hyperglycemia induces macrophage infiltration especially the inflammatory macrophages. The aim of this study is to analyse the effect of hyperglycemia condition on cytokines production by human monocytes-derived macrophages.

Materials and methods: Monocyte cells that obtained from peripheral blood mononuclear cells isolation from donors were incubated for 6 days in 37°C, 5% CO₂. On day 4, the stimulating factors such as lipopolysaccharide (LPS) and interferon (INF)- γ were added to activate monocytes into macrophages. Then on day 6, two doses of glucose; either normal or high doses along with low or high dose of LPS were given for 24 hours, followed by collecting the culture media and cells then stored at -80°C until assayed.

Results: There was significantly different of TNF- α level among groups, with highest level found in group with high dose glucose plus high dose LPS. However, the concentration of IL-6 among groups was not significantly different.

Conclusions: Macrophages treated with high dose glucose plus high dose LPS significantly increased TNF- α production, but not for IL-6.

Key words: glucose, interleukin-6 (IL-6), lipopolisaccharides (LPS), macrophages, TNF- α

INTRODUCTION

Macrophage is part of innate immune cells that responds immediately to any invaders or cell debris. Macrophages have pathogen recognition receptors (PRRs) on its surface that can recognize pathogen associated molecular patterns (PAMPs) or endogenous danger associated molecular patterns (DAMPs) [1]. To bind to those molecules, macrophages equipped with toll like receptors (TLR). NOD like-receptors (NLRs), the HIN-200 receptor family, and RIG-1-like receptor (RLRs) are also types of PRRs that can be found in macrophages which located in the cytoplasm and act as stressor signals when pathogen found in the cytoplasm [2].

Macrophages also have role to maintain tissue homeostasis [3], by polarizing into two types subpopulations, depend on the microenvironment they exposed to [4]. The classical or proinflammatory macrophages, known as M1 and the alternative or anti-inflammatory macrophages as M2 [5]. The imbalance of the M1/M2 ratio occurs in chronic inflammation condition such as insulin resistance, cardiovascular disease, obesity, tumor development, and autoimmune disease [5].

Macrophages infiltration also increases in the metabolic active tissue during over nutrition condition that lead to proinflammatory environment and marked by the increased of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and inducible nitric oxide synthase (iNOS) secretion. In type 2 diabetes mellitus, macrophages involve in progressivity of the disease by altering metabolic pattern [6]. In obese patients, the M1 macrophages infiltrate the enlarged fat cells of adipose tissue and results in production abundance of inflammatory cytokines such as TNF- α and IL-6. These contribute to the decrease of insulin sensitivity and the ability of glucose uptake in fat cells [2].

In the presence of interferon (IFN)- γ and lipopolisaccharide (LPS), macrophage will rapidly activate their glycolytic pathway. Then, the abundance of glucose uptake and utilization lead to the increases of IL-6 and IL-1 β secretion. Hence, the availability of glucose in a tissue may shift macrophages polarization into an inflammatory phenotype [3]. In diabetes, the M1 macrophages persistently lead to diabetic neuropathy by over production of protease, cytokines and reactive oxygen species (ROS). These

molecules induce oxidative stress and degradate myelin and damage neuron regenerative. M1 macrophages may also change pancreas cells functions and cause insulin resistance [7]. The M1 proinflammatory macrophages may produce, release and respond to cytokines and chemokines in its environment such as TNF- α , IL-6, IL-1, and monocyte chemoattractant protein [MCP]-1. On the other hand, if the environments do not need the dominance of proinflammatory macrophages, they adapt to the M2 macrophages. The anti-inflammatory macrophages, M2 then allow the resolution of inflammation. The anti inflammatory cytokines and the growth factors that released by M2 such as the antagonist receptor IL-1, IL-10, and transforming growth factor [TGF]- β , induce the decrease of the pro-inflammatory factors and then reduce the macrophages activation. The diminish of macrophages activation marked the end of acute inflammation. But, if the process is hampered, the resolution of inflammation can not be achieved and chronic inflammation occurs [8].

The hyperglycemic condition may activate monocyte, and increase the expression of pro inflammatory macrophages (M1 pheotype), even if there is no tissue damage or infection [8]. Previous study showed that hyperglycemic mice induced by streptozotocin had vast infiltration of macrophages and developed into a systemic proinflammatory state shown by the increase of IL-6, IL-1b, TNF- α , and IL-8 [9]. An in vitro study on human monocytes and macrophages that exposed to high levels of glucose also underwent into inflammatory macrophages and the same situation apparently also being observed in patients with hyperglycemia [10].

The aim of the study was to analyse the effect of hyperglycemia condition in the environment of the macrophage cell culture.

MATERIAL AND METHODS

Subject recruitment

The blood donors for this study were from three females and one male with age average 20 years old, body weight 64.5 ± 8.44 kg, height 161.5 ± 1.66 cm, and body mass index (BMI) 25.20 ± 3.30 kg/m². All participants were screened for eligible criteria, such as not having any infection and metabolic diseases in one month before and also signed the informed consent after being explained about the study procedures. All procedures of this study were approved by the Ethical Committee of Human

Studies, Faculty of Medicine and Adam Malik General Hospital, Medan. This study was conducted in the Research Laboratory of Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia between July and October 2019.

Isolation of peripheral blood mononuclear cells (PBMCs)

Fifty millilitres of blood was withdrawn from venous and collected in EDTA contained vacutainer tubes. The PBMCs of buffy coat were collected by gradient centrifugation using LymphoprepTM (Stemcell, Axis-Shield, Dundee, UK), according to the manufacturer's instruction.

Isolation of monocytes

Monocytes were isolated from PBMCs by using EasySepTM negative monocyte isolation kit (StemcellTM Technologies, Vancouver, Canada). Briefly, the PBMCs were treated with antibody and platelet removal before selected with magnet beads and incubate in an EasySep Magnet (StemcellTM Technologies, Vancouver, Canada) according to the manufacture's procedures.

Culture of monocytes

Monocytes were cultured in RPMI 1640 (Gibco, Waltham, MA, USA) supplemented with with 10% foetal bovine serum, FBS (Sigma, St Louis, MO, USA), 1% penicillin-streptomycin (Gibco, Waltham, MA, USA) and 1% L-glutamine (Gibco, Waltham, MA, USA) at 37°C in a 5% CO₂ humidified incubator. Recombinant human M-CSF/CSF1 (ABclonal Technology, Woburn, MA, USA) at the concentration of 50 ng/mL was used to direct macrophage differentiation. Cells were incubated for 6 day in 37°C, 5% CO₂. The monocytes seeded in 24 wells culture plates (Iwaki tissue culture treated polystyrene microplate, Asahi Glass Co., Japan). On day 4, fresh media were added with Lipopolysachharide, LPS (10 ng/ml) from E. coli (O26:B6) (Sigma, St Louis, MO, USA) and IFN- γ (50 ng/ml) (StemcellTM Technologies, Vancouver, Canada) to differentiate macrophage into M1 macrophages. On day 6, all media were removed and replaced with serum-free complete media (SF-CM).

Glucose stimulation

The macrophages were divided into five groups, two were supplemented with two doses of glucose (Sigma, St Louis, MO, USA) (7 mM as normal dose, ND-Glu, and 35 mM as high dose, HD-Glu). This concentration is based on the diabetes diagnosis by WHO (11) and doubling the dosage will resemble the diabetes state in patients (8). The other three groups beside having the two doses of glucose also supplemented with LPS (low dose 100 ng/ml, LD-LPS and high doses 2 µg/ml, HD-LPS). The control group was treated with serum-free complete media. The treatments were given for 24 hours after day 6. Then the culture media and cells were harvested and frozen at -80°C until assayed.

Giemsa staining

The coverslips that previously put inside the wells were discarded and left dry in room temperature. Then fixed with 98% methanol for 1 min and bathed with Giemsa (diluted with distilled water, 1:1 vol/vol) for 5 min. The final step, the coverslips were washed with distilled water and left to dry then mounted onto slides. The Giemsa staining of macrophages stimulated with normal dose of glucose is seen in Figure 1 and 2 and in high dose of glucose in Figure 3 and 4, with 10x10 and 10x40 magnification.

Analysis of cytokines production

Human Pico Kine ELISA kits (My BioSource Inc., San Diego, CA, USA) were used to analyse the concentration of TNF-α and IL-6 according to manufacturer's protocols. The assays were performed in duplicate. The intra assay coefficient of variations were < 11%.

Statistical analysis

All data are presented as mean ± standard deviation. All data were normally distributed after analysed using Shapiro Wilk test, then were analysed with one way ANNOVA and followed by Bonferroni post hoc test. The p value < 0.05 was considered statistically significant. All data were statistically analysed using SPSS software version 17.0.

RESULTS

The production of TNF- α

Test of variances homogeneity with Leven test showed that there is significant differences in variance with p value = 0.001, then one way ANOVA Welch test showed that the data is significantly different among groups with p value = 0.004 (p value < 0.05). The Robust Test of Equality of Means showed the p value = 0.29 (p value > 0.05) (equals variances assumed), so the analysis was continued with the post hoc test Bonferroni to find which groups are significantly different.

As can be seen in Tabel 1, the level of TNF- α were significantly different among groups with p = 0.004, group treated with normal dose of glucose had lowest TNF- α concentration (40.67 ± 9.94 pg/mL), while in group treated with high dose of glucose plus high dose of LPS had highest TNF- α concentration (2150.22 ± 1379.77 pg/mL).

This study showed that macrophages treated with normal dose or high dose of glucose and/or stimulated with low dose or high dose of LPS had higher concentration of TNF- α compared to the treated macrophages. Then, after being analysed with the Bonferroni post hoc test, it was shown that there are significantly differences of TNF- α concentration within groups; Control vs HD-Glu+HD-LPS (mean differences (MD) = -2107.34 pg/mL; p value = 0.015), ND-Glu vs HD-Glu+HD-LPS (MD = -1943.53 pg/mL; p value = 0.009), and HD-Glu vs HD-Glu+HD-LPS (MD = -2095.69 pg/mL, p value = 0.002). It means that high dose of glucose plus high dose of LPS resulted in an increase of TNF- α production (Table 2).

The production of IL-6

This study showed that the lowest concentration of IL-6 were found in group treated with high dose of glucose (43.37 ± 16.29 pg/mL), meanwhile the highest concentration was found in group with high dose of glucose plus high dose of LPS (59.66 ± 16.72 pg/mL). But after being analysed with one way ANOVA test, the concentrations of IL-6 were not significantly different (p value=0.742). see Table 3.

The macrophages staining

This study also performed the Giemsa staining of macrophages with normal dose and high dose glucose. The figures are shown with 10x10 and 10x40 magnification. see Figure 1, 2, 3, and 4.

DISCUSSION

We observed that the highest level of TNF- α was found in the HD-Glu + HD-LPS group, and the lowest was found in control group (p value = 0.004). This finding is similar to previous study that compared the production of proinflammatory cytokines in macrophages that stimulated with LPS to the non-stimulated macrophages. The previous study showed that the production of TNF- α , MCP-1, IL-6 and IL-1 β were significantly higher in stimulated macrophages compared to control [7]. Previous study also showed that THP-1 macrophage cultured in high glucose concentration (15mM) with or without LPS stimulation displayed higher concentration of TNF- α compared to normal glucose (5 mM) or osmotic control group [8]. Furthermore, activated macrophages that stimulated with hyperglycemia and hypoxia condition were shown to upregulate TNF- α , IL-6 and IL-1 gene expression [11]. Another study on diabetic rats induced by streptozotocin also shown an increase of TNF- α and overexpression of inflammatory genes, such as TNF- α , COX-2 and iNOS [12].

Differences of TNF- α concentration was observed among groups; Control vs HD-Glu+HD-LPS, ND-Glu Vs HD-Glu+HD-LPS, and HD-Glu vs HD-Glu+HD-LPS with each p value were 0.015, 0.009, and 0.002 (p value<0.05), respectively. The macrophages with high dose glucose plus high dose LPS were significantly produce higher TNF- α compared to the serum free medium (control), or to the macrophages with normal or high dose glucose.

The tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), or the recognition of LPS are the Th1 (T helper 1) cytokines that can trigger the polarization of proinflammatory M1 macrophages. The activity of these M1 macrophages will then increase the expression of IL-12, IL-23, and the production of TNF, IL-1 β and IL-6, and also the reactive oxygen species (ROS) via activation of NADPH oxidase system. The function of this action is to remove pathogen during infection that results in ROS-mediated tissue damage and decline wound healing [13]. It also has been known that the circulating LPS bind with LPS binding protein (LBP) and caught by CD14 surface scavenging receptor. This signal activates TLR that initiates the phosphorylation cascade mediated by different kinase enzymes. The result of this signal is releasing transcription factor NF κ B from its inhibitor I κ B and translocate to the nucleus. The

effect of this cascade is increasing the expression of inflammatory genes such as TNF, antibiotic peptides and NADPH oxidase that produce the reactive oxygen intermediate [14].

We observed that the highest level of IL-6 was found in HD-Glu + HD-LPS and the lowest was found in HD-Glu, but the differences were not significant (p value > 0.05). This study showed that the IL-6 production was not changed by glucose and/or LPS stimulation. This result is in contrary to previous study that analysed the differences in cytokine production among type 2 diabetes patients and Diabetic Peripheral Neuropathy (DPN) patients, the macrophages from patients with type 2 diabetes had significantly produce higher level of IL-6 when compared to control and DPN patients [7]. Another study also showed that THP-1 macrophages produced higher level of IL-6 when stimulated with high glucose and LPS, in compare to control group. The concentration of normal glucose in that study was 5mM and the high glucose was 15mM [8]. It means that M1 macrophages are more metabolically active in hyperglycaemic conditions, and promote obesity associated insulin resistance [15]. The different of this finding to those studies is the dose being used. In addition, the dramatically increased of the IL-6 gene expression was seen after 17 hours activation and stimulated with hyperglycemic along with hypoxia [11], meanwhile in this study the concentration of IL-6 was analysed after 24 hours stimulated. Similar results also found in the previous study of monocytes-derived macrophages exposed to high glucose (15mM) that showed no significant differences of TNF- α production when compared to the normal glucose (5mM) or hyperosmotic (glucose free) conditions [10].

The limitation of this study is we did not perform the immunophenotypic assessment that is useful to identify and count the percentage of cells. Anyhow, we identify the cells using the cytomorphology and the number of cells with improved Neubauer chamber.

CONCLUSIONS

Finally, this study concluded that macrophages which stimulated with high dose of glucose plus high dose of LPS significantly increased the TNF- α production. On the other hand, different dosages of glucose and LPS stimulation to the macrophages cell cultures did not change the production of IL-6.

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Table 1. The TNF- α level among groups

Groups	TNF-α (pg/mL)	p value
Control	42.88 \pm 22.85	0.004*
ND-Glu	40.67 \pm 9.94	
HD-Glu	54.43 \pm 26.93	
ND-Glu+HD-LPS	915.09 \pm 804.02	
HD-Glu+LD-LPS	979.49 \pm 8226.84	
HD-Glu+HD-LPS	2150.22 \pm 1379.77	

Values are Mean \pm SD (n=4). Control (serum-free media only), ND-Glu (normal dose glucose, 7 mM), HD-Glu (high dose glucose, 35 mM), LD-LPS (low dose LPS, 100 ng/mL), HD-LPS (high doses LPS, 2 μ g/mL). * pvalue<0.05 (The one one way ANOVA test).

Table 2. The post hoc test of TNF- α level among groups

Groups	Mean	95% Confidence Interval		p value
	differences	Lower Bound	Upper Bound	
Control vs ND-Glu	-163.81	1852.16	1524.53	1.00
Control vs HD-Glu	-11.65	1661.18	1637.88	1.00
Control vs HD-Glu+LD-LPS	-936.61	2841.33	968.09	1.00
Control vs ND-Glu+HD-LPS	-872.21	2776.92	1032.50	1.00
Control vs HD-Glu+HD-LPS	-2107.34	4012.05	202.63	0.015*
ND-Glu vs HD-Glu	-152.16	1241.94	1546.27	1.00
ND-Glu vs HD-Glu+LD-LPS	-772.80	2461.15	915.54	1.00
ND-Glu vs ND-Glu+HD-LPS	-708.40	2396.74	979.95	1.00
ND-Glu Vs HD-Glu+HD-LPS	-1943.53	3631.87	255.18	0.009*
HD-Glu vs HD-Glu+LD-LPS	-924.96	2574.49	724.56	1.00
HD-Glu vs ND-Glu+LD-LPS	-860.56	2510.09	788.97	1.00
HD-Glu vs HD-Glu+HD-LPS	-2095.69	3745.22	446.16	0.002*
HD-Glu+LD-LPS vs ND-	64.41	1840.30	1968.12	1.00

Glu+HD-LPS

HD-Glu+LD-LPS vs -1170.72 3075.43 733.99 1.00

HD-Glu+HD-LPS

ND-Glu+HD-LPS vs -1235.13 3139.84 669.58 1.00

HD-Glu+HD-LPS

Control (serum-free media only), ND-Glu (normal dose glucose, 7 mM), HD-Glu (high dose glucose, 35 mM), LD-LPS (low dose LPS, 100 ng/mL), HD-LPS (high doses LPS, 2µg/mL). * pvalue<0.05 (Bonferroni post hoc test)

Table 3. The IL-6 level among groups

Groups	IL-6 (pg/mL)	p value
Control	55.16 ± 23.19	0.742
ND-Glu	49.52 ± 29.28	
HD-Glu	43.37 ± 16.29	
ND-Glu+HD-LPS	53.06 ± 20.45	
HD-Glu+LD-LPS	52.08± 23.85	
HD-Glu+HD-LPS	59.66 ± 16.72	

Values are Mean ± SD (n=4). Control (serum-free media only), ND-Glu (normal dose glucose, 7 mM), HD-Glu (high dose glucose, 35 mM), LD-LPS (low dose LPS, 100 ng/mL), HD-LPS (high doses LPS, 2µg/mL).

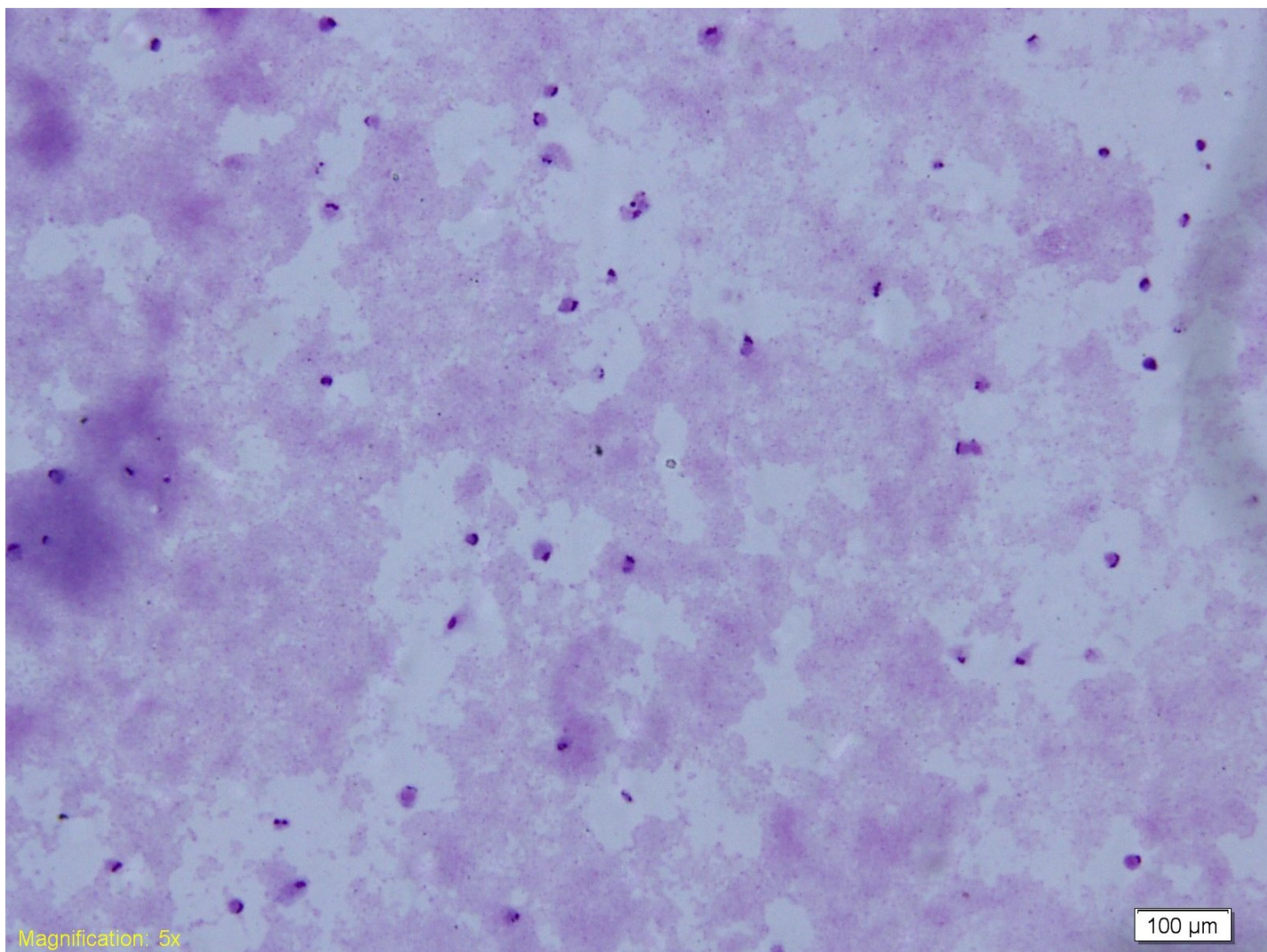
* pvalue<0.05 (The one one way ANOVA test).

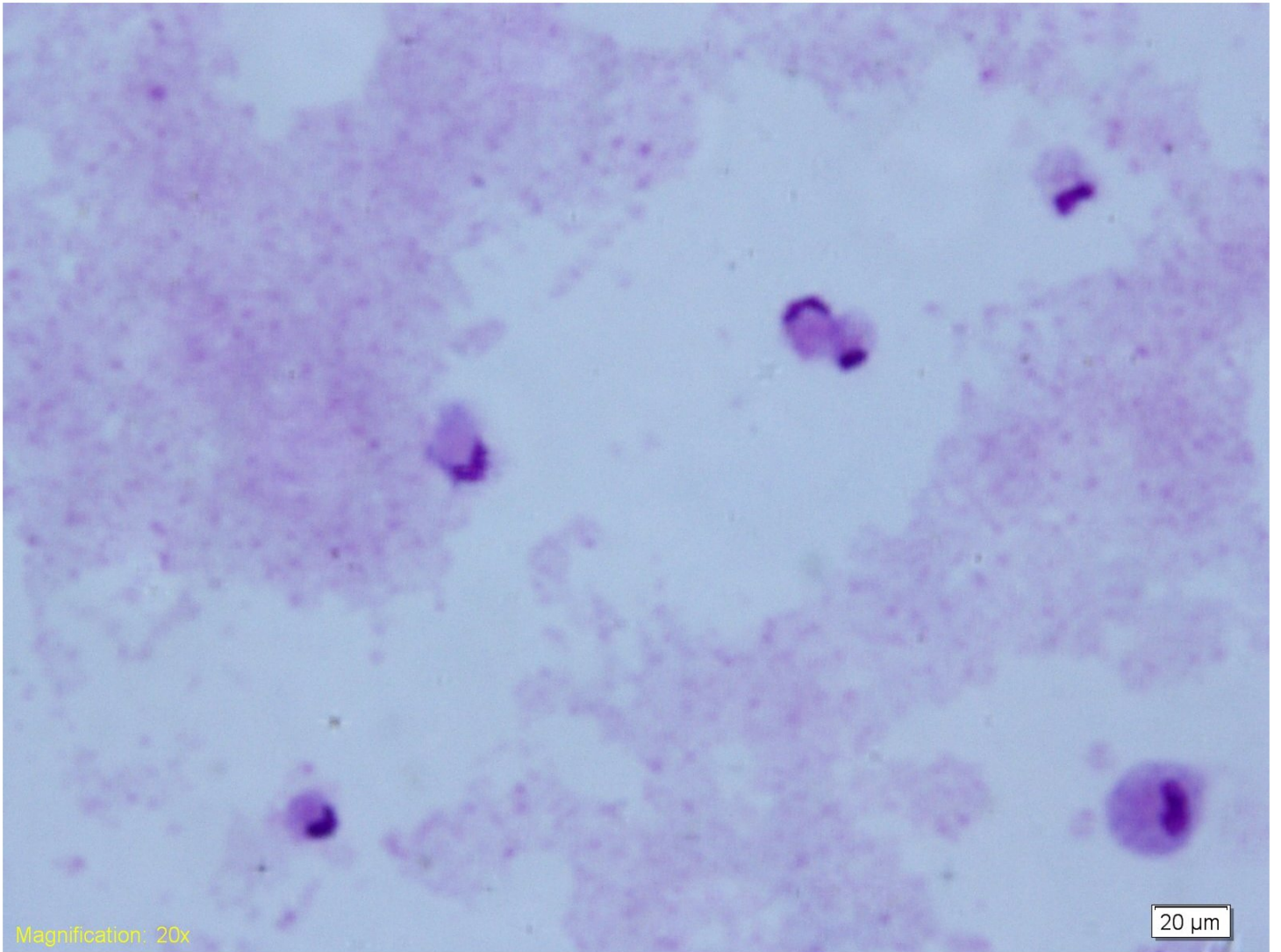
Figure 1. Normal glucose 10 × 10.

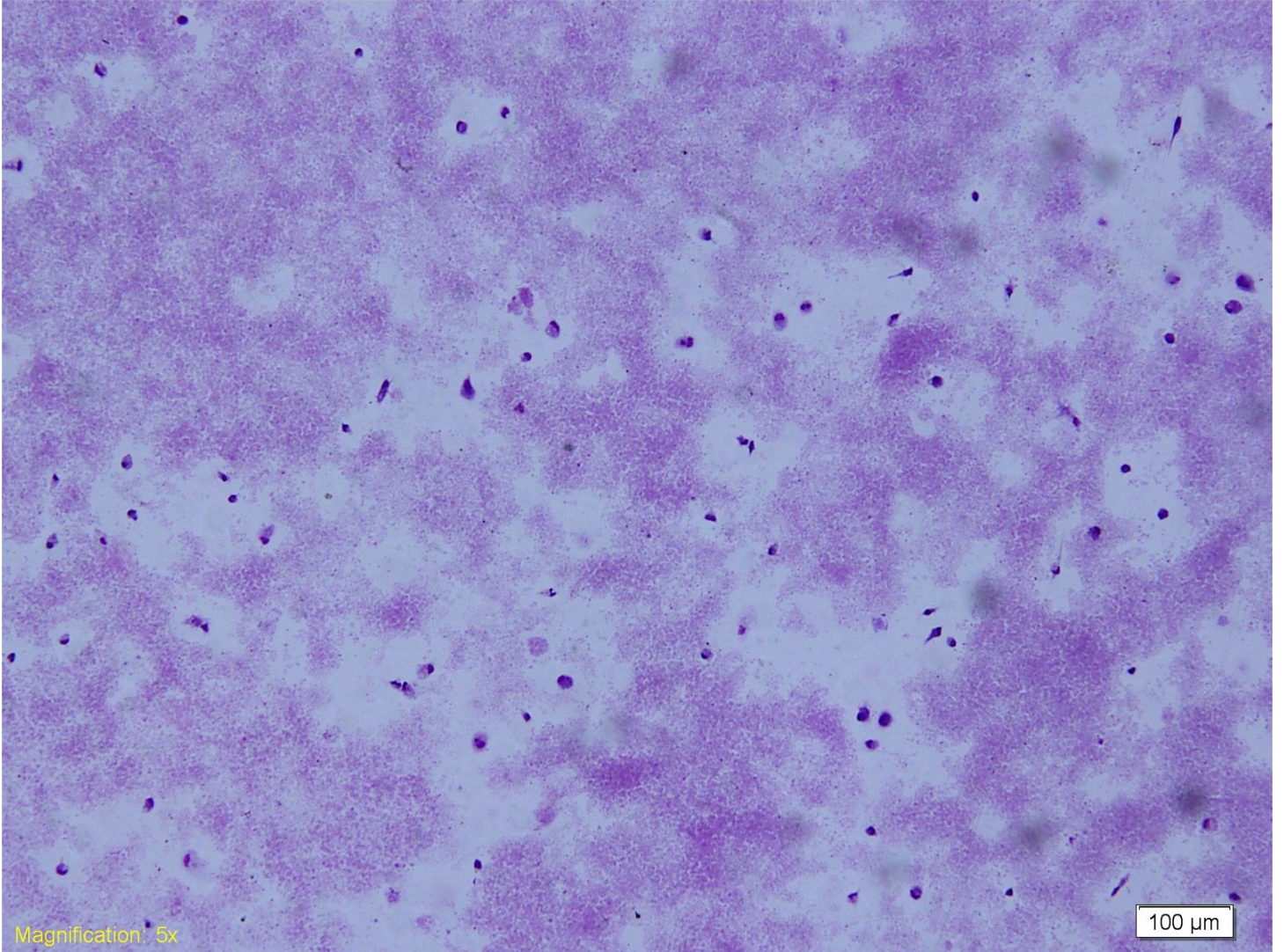
Figure 2. Normal glucose 10 × 40.

Figure 3. High glucose 10 × 10.

Figure 4. High glucose 10 × 40.







Magnification: 5x

100 μm

